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Dietary protein mediates a trade-off between larval survival and the development of male secondary sexual traits

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Summary

1. Increased protein intake by adults typically enhances reproduction and shortens life, but much less is known about the effects of protein intake prior to reproductive maturity. In particular, it remains unclear whether dietary protein mediates a trade-off between survival and the development of reproductive traits, especially in males.

2. We used the nutritional geometry approach to investigate the effects of 25 replicated larval diets varying in protein and carbohydrate content on larval performance and the development of adult male morphology in the neriid fly *Telostylinus angusticollis*.

3. We found that body size increased with carbohydrate and especially protein content in the larval diet. Moreover, after controlling for body size, dietary protein strongly enhanced the expression of those male morphological traits that appear to be most directly targeted by sexual selection, while having no effect on the expression of other traits. In sharp contrast, egg-to-adult viability decreased steeply with increasing protein content.

4. Thus, protein intake during the larval stage mediates a trade-off between the expression of male secondary sexual traits and the probability of survival to adult emergence.

Key-words: body shape, condition dependence, dietary restriction, larval diet, nutritional geometry

Introduction

It has been known for nearly a century that moderate restriction of dietary nutrients (dietary restriction) results in extended longevity and reduced reproduction in a broad range of animal species (Mair & Dillin 2008; Fontana, Partridge & Longo 2010; Nakagawa *et al.* 2012). Recent studies have established that, at least in some taxa, this apparent trade-off is mediated by dietary protein: increased protein content in the adult diet enhances egg output by females, but at the cost of reduced longevity (Lee *et al.* 2008; Maklakov *et al.* 2008; Je *et al.* 2009). A nutrient-rich adult diet may reduce survival because of the immediate and latent costs of elevated physiological and metabolic processes associated with investment in reproduction, such as the production of reactive oxygen species (Mohanty *et al.* 2002; Sanz, Caro & Barja 2004; Caro *et al.* 2009) or down-regulation of cellular repair pathways (Tarry-Adkins *et al.* 2007; Martin-Gronert *et al.* 2008), or because of the toxicity of an unbalanced diet containing excess protein (Piper & Partridge 2007). However, research on dietary protein restriction has focused largely on the effects of the adult diet on females. Effects of dietary protein restriction on males, and especially during development, remain poorly understood.

In insects, restricted access to dietary protein during the growth and development phase of the life cycle typically results in reduced body size (Gebhardt & Stearns 1988; Ottenheim & Holloway 1995; Tu & Tatar 2003; Colasurdo, Gelinas & Despland 2009), but effects on viability vary across taxa and studies, apparently reflecting nonlinear responses to protein concentration (Gebhardt & Stearns 1988; Nestel, Nemny-Lavy & Chang 2004; Hahn 2005; Anagnostou, Dorsch & Rohlfs 2010; Andersen *et al.* 2010; Lee & Roh 2010). Nonetheless, there is some evidence that, as in the adult diet, dietary protein during development mediates trade-offs between fitness components. Experiments on the dipteran *Drosophila melanogaster* and the moth *Spodoptera littoralis* show that individuals reared on a high-protein diet tend to exhibit an enhanced capacity to mount an immune defence as larvae or adults, apparently because dietary protein is required for the

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biosynthesis of lysozymes and other antimicrobial peptides (Vass & Nappi 1998; Fellous & Lazzaro 2010; Cotter *et al.* 2011). However, *S. littoralis* caterpillars reared on a protein-rich diet may perform poorly in terms of growth and, in the absence of an immune challenge, may suffer reduced survival in comparison with individuals provided with lower dietary protein (Cotter *et al.* 2011). In *Drosophila melanogaster* males, a protein-rich larval diet confers increased capacity to resist heat and desiccation, but reduces egg-to-adult viability (Andersen *et al.* 2010). The picture is complicated further by the potential for sex-specific effects. For example, in *D. melanogaster*, a reduced protein:carbohydrate (P:C) ratio increases egg-to-adult viability for males but reduces it for females (Andersen *et al.* 2010), suggesting that dietary protein may have very different consequences for male and female fitness during development, just as it does during the adult stage (Maklakov *et al.* 2008).

In many animals, structures that play important roles in reproduction grow and develop prior to the adult stage. In particular, in holometabolous insects, the precursors of adult tissues (imaginal discs) grow inside the larval body and develop into adult reproductive structures during metamorphosis (Hemming 2003). A special class of adult reproductive traits that has been of considerable interest to evolutionary biologists are secondary sexual traits, which are typically expressed exclusively by males, but also found in females in some species (Clutton-Brock 2009). Secondary sexual traits sometimes exhibit extreme exaggeration, and the size of such structures can have enormous consequences for male fitness – with larger traits enhancing mating success but reducing viability (Andersson 1986, 1994; Emlen & Nijhout 2000) – but the developmental mechanisms involved in their expression are complex. Male secondary sexual traits are typically strongly condition-dependent, such that individuals in high condition express relatively larger traits than individuals in low condition (Cotton, Fowler & Pomiankowski 2004; Bonduriansky 2007). The strong condition dependence of such traits may reflect the heightened sensitivity of the imaginal tissues that develop into these structures to insulin/insulin-like growth factor (IGF) signalling (Emlen *et al.* 2012). In a broad range of animal taxa, this metabolic signalling pathway regulates the rate of somatic tissue growth by linking the availability of dietary nutrients to cell growth and proliferation (Britton & Edgar 1998; Brogiolo *et al.* 2001; Britton *et al.* 2002; Ikeya *et al.* 2002; Teleman 2009; Tennessen & Thummel 2011). In *Drosophila*, it has been shown that this pathway responds primarily to the availability of dietary protein (Britton & Edgar 1998; Britton *et al.* 2002). Dietary proteins also furnish key building blocks for tissue growth (Teleman 2009; Harrison, Woods & Robers 2012). Thus, we expected that the imaginal tissues that develop into adult secondary sexual traits would be especially sensitive to protein abundance in the larval diet and predicted that a protein-rich larval diet would enhance the expression of adult secondary sexual traits in males.

Nutritional geometry, a recently-developed approach to research on the effects of diet composition, offers a promising way to investigate the effects of dietary protein on trait expression and fitness (Simpson & Raubenheimer 2007; Lee *et al.* 2008; Maklakov *et al.* 2008). Levels of two key diet components, such as the macronutrients protein and carbohydrate, are manipulated simultaneously to produce a range of diets varying in nutrient ratio as well as net concentration, resulting in a set of 'nutritional rails'. Each nutritional rail represents one macronutrient ratio in a range of concentrations (Fig. 1). The effects of variation in each nutrient as well as their interaction can then be visualized as a response surface, allowing for the detection of nonlinear and combinatorial effects. The nutritional geometry approach has been used to demonstrate the key role of protein in mediating the trade-off between survival and reproduction in adult female flies (Lee *et al.* 2008; Fanson & Taylor 2011b), the trade-off between larval immunity and performance in moth caterpillars (Cotter *et al.* 2011), and the sex-specific effects of protein on reproduction in crickets (Maklakov *et al.* 2008).

We used the nutritional geometry approach to investigate the effects of protein and carbohydrate in the larval diet on growth and viability of the neriid fly *Telostylinus angusticollis*. This species exhibits pronounced developmental plasticity to nutrient concentration in the larval diet: larvae reared on a nutrient-rich medium develop into much larger adults, with relatively larger secondary sexual traits in males, than larvae reared on a lower nutrient concentration (Bonduriansky 2007). However, the separate effects of the macronutrients (protein and carbohydrate) in the larval diet have not been investigated before. We reared larvae on 25 replicated diets varying in P:C ratio and total nutrient concentration and investigated diet effects on egg-to-adult viability and adult male body size and shape.

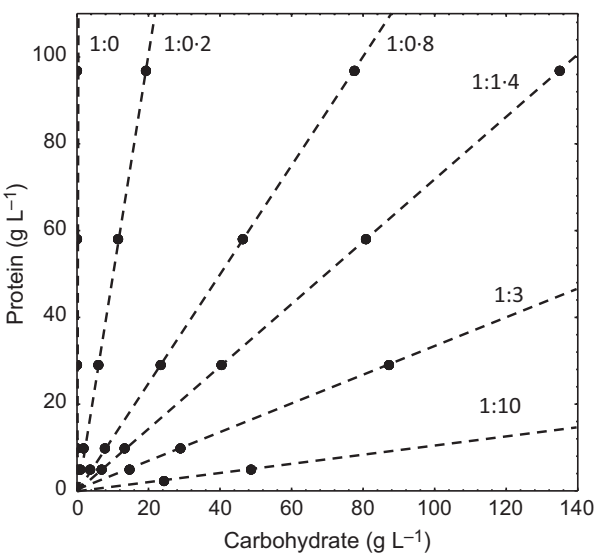

Fig. 1. Nutritional rails representing six P:C ratios used in the experiment. Points along the rails represent amounts of protein and carbohydrate (g) mixed with 1 L of dry cocopeat and 800 mL of water to prepare the experimental diets.

We were particularly interested in the effects of dietary macronutrients on the expression of the most exaggerated secondary sexual trait – head shape. Males use their greatly enlarged and elongated heads and antennae as weapons in combat with rivals for control of territories and access to females (Bonduriansky 2006), and experiments show that males with more elongated antennae (relative to body size) tend to defeat rivals in male–male combat and achieve mating more quickly in male–female interactions (C. Fricke, M.I. Adler, R.C. Brooks and R. Bonduriansky, unpublished data). The male head is also the most sexually dimorphic and condition-dependent structure in this species (aside from the genitalia), and male head capsule and antenna length are the only morphological traits whose static allometry is affected significantly by nutrient availability during development (Bonduriansky 2007). For comparison, we also examined diet effects on leg and wing dimensions, which appear to play less direct roles in male sexual competition (Bonduriansky 2006; C. Fricke, M.I. Adler, R.C. Brooks and R. Bonduriansky, unpublished data), and exhibit less pronounced sexual dimorphism and weaker condition dependence (Bonduriansky 2007). Larval diet effects on adult female body size and shape will be presented in a separate paper.

Materials and methods

EXPERIMENTAL ANIMALS

The *Telostylinus angusticollis* flies used in this experiment were derived from approximately 100 adult individuals collected from the trunks of *Acacia longifolia* trees in Fred Hollows Reserve, Sydney, and reared as large, outbred populations in the laboratory for 2–4 generations on standardized larval diets. The parents of the experimental flies were obtained from these populations as newly emerged adults, separated by sex, and housed in same-sex groups for 10 days. These flies were then randomly assorted into mixed-sex groups, each containing 2 or 3 individuals of each sex, and housed in 1 L cages with three Petri dishes of oviposition medium to collect eggs for the experimental manipulations. Twelve groups of parents were composed of flies derived from populations reared in captivity for two generations, and 10 groups were composed of flies derived from populations reared in the laboratory for four generations. All Petri dishes were checked daily for eggs, and the oviposition medium was replaced every 2–3 days.

EXPERIMENTAL DIETS

The 25 experimental diets were prepared by adding varying amounts of brown sugar and soy protein powder (Nature's Way brand; Pharm-a-care Pty. Ltd., Warriewood, NSW, Australia) to 1 L of dry cocopeat (Galuku Pty. Ltd., Sydney, NSW, Australia) hydrated with 800 mL of purified water. The brown sugar in these diets replaced the molasses and barley malt used in previous diet manipulation experiments on this species (Bonduriansky 2007, 2009; Bonduriansky & Head 2007). Pilot studies revealed no noticeable effect of this replacement on larval performance or adult morphology. The brown sugar used in our experimental diets consists (by weight) of 98% sugars (including sucrose, fructose, and other sugars), 0·2% protein, and minerals (mainly sodium). The soy protein powder consists of 18 amino acids (Alanine, Arginine, Aspartic Acid, Cysteine, Glutamic Acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, Valine). Although we refer to effects of carbohydrate and protein throughout, our experimental diets obviously consist of one particular type of each of these macronutrients, and (as with all diet manipulation experiments) it would be useful to replicate this study using alternative types of carbohydrate and protein to verify the generality of our results. Cocopeat itself has no nutritional value for fly larvae. Diet mixtures were mixed thoroughly, and one-eighth of each diet mixture (determined by weight) was transferred to each of five 200-mL containers (experimental replicates) and frozen at −20 °C until the day of use. The experimental diets represented six ratios of protein (P) to carbohydrate (C), with several nutrient concentrations for each ratio (Fig. 1). The exact composition of each diet is shown in Table 1. The standard 'rich' and 'poor' larval diets used in previous experiments on this species (Bonduriansky 2007, 2009; Bonduriansky & Head 2007) are near the centre of the range of diets used in the present study.

Experimental procedure

A total of 20 eggs were transferred on the same day to each of the 125 replicate containers (five containers for each of 25 larval diets). Eggs were randomly allocated to replicate containers to minimize effects of environmental variation or parental age, and the eggs transferred to each replicate were sourced from at least five parental groups to reduce effects of genetic variation. All replicate containers were placed in a controlled-environment chamber at a temperature of 27 °C and 50% humidity. At 9 and 12 days in the environment chamber, 24 and 16 mL of water respectively were added to each container to keep the larval medium moist, and the larval medium was thoroughly mixed. After 21 days (i.e. when most larvae had pupated), all containers were removed from the environment chamber and placed into 2 L cages with a layer of moist cocopeat on the bottom to allow for adult emergence. The adults in each container were provided with water and sugar. Ten days after the first emergence, once most adults had emerged, five offspring of each sex (where possible) were randomly sampled from each container for morphometric analysis and frozen at −20 °C. Fifteen days after the first emergence, the total number of adult flies was recorded for each replicate to calculate egg-to-adult viability.

Table 1. Amounts (g) of soy protein powder and brown sugar used to prepare each of the 25 experimental diets

	P:C ratio					
	1:0	1:0·2	1:0·8	1:1·4	1:3	1:10
Nutrient	Amount					
Protein	110·1	110·1	109·9	109·8	32·8	5·4
Sugar	0	19·8	79·1	137·8	89	49·4
Protein	66·1	66	66	65·9	10·9	2·7
Sugar	0	11·9	47·5	82·7	29·7	24·7
Protein	33	33	33	32·9	5·5	
Sugar	0	5·9	23·7	41·3	14·8	
Protein	11	11	11·1	11		
Sugar	0	2	7·9	13·8		
Protein	5·5	5·5	5·5	5·5		
Sugar	0	1	4	6·9		

Corresponding protein: carbohydrate ratios are shown at the top.

Morphometric data

Each individual's wings and all legs on one side of the body were removed, mounted on microscope slides and photographed under a Leica MS5 stereoscope fitted with a DFC420 digital camera. The body was then photographed in dorsal and lateral view. From these images, we used ImageJ software (Rasband 1997-2012) to measure the following traits: thorax length (TL), head capsule length, head capsule width, antenna length, fore-tibia length, mid-tibia length, hind tibia length and wing length (length of the R_{4+5} vein length from the r-m cross-vein to the wing margin). Wing length was determined from the mean for both wings, or if one wing was damaged, from the length of the intact wing.

Analysis

Replicate containers are the appropriate observational units for testing treatment effects in our design, so all analyses were carried out on replicate container means (Quinn & Keough 2002). Note that even though our design does not include an equal number of concentrations along each nutritional rail (Fig. 1), each of the 25 diets used in this experiment was replicated five times. For each response variable, we fitted general linear (multiple regression) models with amounts of protein (P) and carbohydrate (C), and their quadratic terms (P^2, C^2) and product (P × C), as continuous predictors. The assumptions of multiple regression analysis (in particular, normal distribution of residuals) were met for each dependent variable. Effects were represented as standardized partial regression coefficients (β). Response surfaces were visualized as thin-plate splines projected in two dimensions (P and C), with colour used to represent the third (response) dimension. We examined effects on egg-to-adult viability (number of adults that emerged from a replicate out of 20 eggs transferred), male body size and the relative sizes of male head, leg and wing traits. Egg-to-adult viability was based on data for both sexes because it was not possible to determine the sex of individuals that failed to survive. The viability analysis is based on five replicates for each of the 25 diets, but replication within diets varies in the morphology analyses because not all replicates yielded adult males, and some traits could not be measured on some males.

Body size was quantified as the individual score on the first principal component (PC1) from principal component analysis performed on the correlation matrix for all male morphological traits. For morphological traits, PC1 typically represents a body size axis on which all traits load with similar sign, and individual scores on PC1 therefore represent a comprehensive index of body size variation (Berner 2011). All trait loadings on PC1 exceeded 0·98.

To examine diet effects on the expression of male head, leg and wing dimensions relative to body size, we first carried out a multivariate analysis of variance (MANOVA) on data on all morphological traits. To further explore the effects detected by the MANOVA, we then carried out separate analyses for each trait. We included body size as a covariate in the models to account for the strong correlation between overall body size and the sizes of separate traits. Because all traits were included in the matrix on which principal component analysis was carried out, PC1 scores do not provide an unbiased index of body size for analysis of variation in relative (size-corrected) trait expression (Berner 2011). Thus, we used thorax length (TL) as the index of body size in these analyses. TL loaded very strongly on PC1 (loading > 0·99) and therefore represents a good single-trait index of overall variation in body size. Thorax length was used as an index of body size in our previous analyses of morphological variation in this species (Bonduriansky 2006, 2007, 2009; Bonduriansky & Head 2007) and is widely used as a body size index in other species of Diptera (Partridge & Fowler 1993; Partridge *et al.* 1999; David *et al.* 2000; Berglund *et al.* 2008; Valtonen *et al.* 2012).

The scaling of traits with TL is linear in most cases, except for male head capsule length, antenna length and wing length, which exhibit slightly nonlinear scaling that is well described by a quadratic function of TL (analysis not shown). However, qualitatively similar results are obtained with or without the inclusion of TL^2 in models for these traits, and we show results for models without TL^2 to minimize colinearity of independent variables. However, to visualize the effect of protein on the body size-corrected expression of male head elongation, we plotted standardized residual total head length (head + antenna) against protein content in the larval diet, based on residuals from quadratic regression of total head length on thorax length ($R^2 > 0{\cdot}98$).

Results

VIABILITY

Egg-to-adult viability varied among replicates from 0 to 95% and was affected by carbohydrates and protein, as well as a quadratic effect of protein and marginally non-significant protein × carbohydrate interaction (Table 2; Fig. 2). Viability decreased strongly with increasing protein in the larval diet (Fig. 3).

BODY SIZE

Body size, quantified as the individual score on the first principal component (PC1), exhibited positive linear effects and negative quadratic effects of both carbohydrate and protein, but no significant interaction effect (Table 2; Fig. 4). Body size peaked at intermediate concentrations of both carbohydrate and protein in the larval diet and declined slightly at the highest concentrations. Qualitatively identical results (i.e. significant positive linear and

Table 2. Effects of dietary carbohydrate (C) and protein (P) and their squares and product on egg-to-adult viability (proportion emerged out of 20) and adult male body size

	Viability†		Body size‡	
Effect	β	*F*	β	*F*
C	**0·49**	6·31*	**1·19**	46·37****
P	**−1·21**	20·32***	**1·60**	62·26****
C^2	0·05	0·03	**−0·69**	6·15*
P^2	**0·62**	4·90*	**−1·12**	22·83****
C × P	−0·49	3·69	−0·31	1·50

Standardized partial regression coefficients (β) and *F*-ratios are shown for each effect. Significant coefficients are highlighted in bold.
$^{*}P < 0·05$; $^{**}P < 0·01$; $^{***}P < 0·001$; $^{****}P < 0·0001$.
†Error d.f. = 118, whole-model adjusted $R^2 = 0·48$, $P < 0·0001$.
‡Error d.f. = 86, whole-model adjusted $R^2 = 0·69$, $P < 0·0001$.

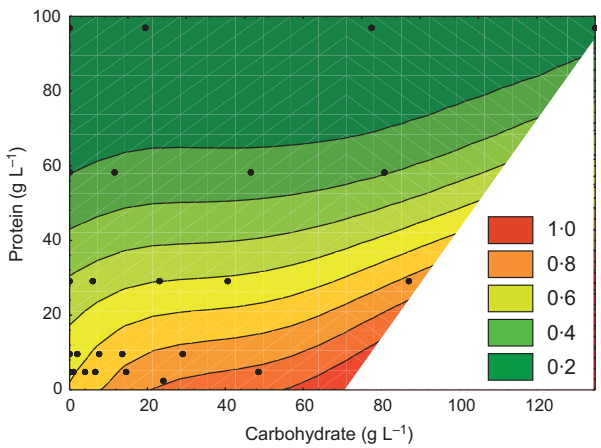

Fig. 2. Response surface for egg-to-adult viability as a function of protein and carbohydrate content (g L^{-1}) in the larval diet. Values of the response variable are indicated by colour, based on thin-plate spline projection fitted to replicate means. Points represent the experimental diets.

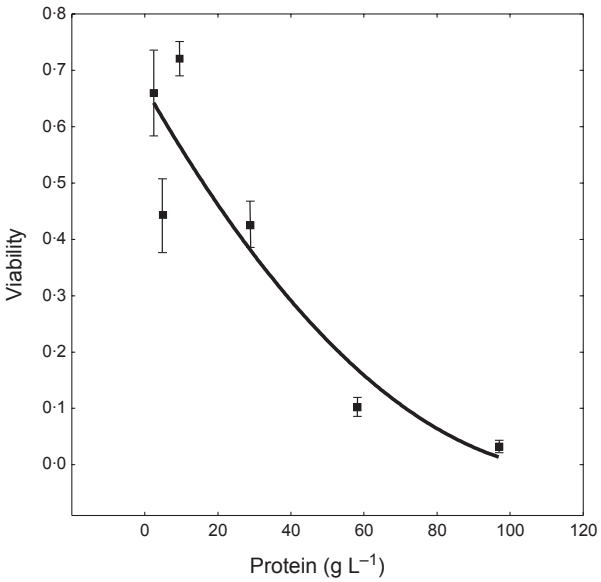

Fig. 3. Egg-to-adult viability (number of adults as a proportion of the number of eggs transferred) as a function of protein content in the larval diet (g L^{-1}). Means for each level of protein are shown, with bars representing the standard error of the mean of replicate means. A quadratic function is fitted to the data ($Y = 0·67 - 0·011X + 0·000047X^2$).

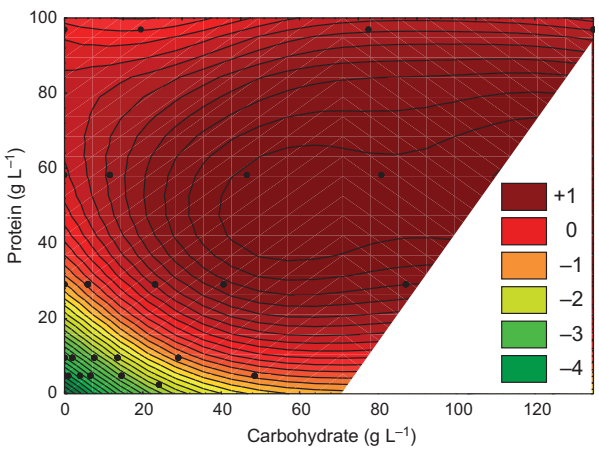

Fig. 4. Response surface for male body size as a function of protein and carbohydrate content (g L^{-1}) in the larval diet. Values of the response variable (in standard deviations from the grand mean) are indicated by colour, based on thin-plate spline projection fitted to replicate means. Points represent the experimental diets.

negative quadratic effects of protein and carbohydrate without significant interaction, not shown) were obtained in univariate analyses for thorax length and all other morphological traits. Response surfaces for the absolute sizes of all morphological traits were also shaped similarly to the PC1 response surface (Fig. 4), with a rapid increase from low to moderate nutrient concentrations, a broad plateau at intermediate nutrient concentrations and a slight decline at the highest nutrient concentrations.

BODY SHAPE

Multivariate analysis of variance (MANOVA) with all head, leg and wing dimensions as response variables and thorax length included as a covariate indicated a significant linear effect of protein (Pillai trace = 0·471, $F_{7,79} = 10·06$, $P < 0·0001$) and quadratic effect of protein (Pillai trace = 0·335, $F_{7,79} = 5·68$, $P < 0·0001$) on relative trait size, but no effects of carbohydrate nor a significant carbohydrate × protein interaction (Pillai trace < 0·14, $F_{7,79} < 1·8$, $P > 0·1$). To determine which traits contributed to this result, we investigated macronutrient effects on each trait separately. The relative dimensions of the male head (head capsule length and width, antenna length) all exhibited positive linear effects of dietary protein, and head capsule length also exhibited a negative quadratic effect of protein, while head capsule width was subject to a protein × carbohydrate interaction (Table 3). None of these traits exhibited linear or quadratic effects

Table 3. Effects of carbohydrate (C) and protein (P) content in the larval diet and their squares and product on male head capsule length and width and antenna length

	Head length		Head width		Antenna length	
Effect	β	*F*	β	*F*	β	*F*
C	−0·03	0·43	0·05	1·07	−0·02	0·30
P	**0·21**	17·12****	**0·12**	4·53*	**0·19**	16·50***
C^2	0·03	0·26	0·09	2·41	0·04	0·71
P^2	**−0·18**	13·68***	−0·04	0·61	**−0·14**	9·06**
C × P	0·03	0·52	**−0·17**	10·54**	−0·01	0·04
TL	**0·94**	2153·17****	**0·94**	1807·73****	**0·94**	2373·59****

Thorax length (TL) was included in all models as an index of body size. Standardized partial regression coefficients (β) and *F*-ratios are shown for each effect. Significant coefficients are highlighted in bold.
Error d.f. = 85, whole-model adjusted $R^2 > 0{\cdot}97$, $P < 0{\cdot}0001$.
$^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

Table 4. Effects of carbohydrate (C) and protein (P) content in the larval diet and their squares and product on male leg and wing dimensions

	Foretibia length		Mid-tibia width		Hind tibia length		Wing length	
Effect	β	*F*	B	*F*	β	*F*	β	*F*
C	0·02	0·41	−0·03	0·89	−0·02	0·46	−0·05	1·07
P	0·03	0·78	−0·01	0·05	−0·08	3·28	−0·10	2·77
C^2	−0·01	0·07	0·04	0·95	0·04	0·69	0·12	3·09
P^2	0·00	0·01	0·01	0·13	0·06	1·77	0·10	2·81
C × P	0·00	0·00	0·01	0·03	0·02	0·16	−0·09	2·64
TL	**0·98**	**5212·62******	**0·99**	**4456·27******	**1·01**	**3418·42******	**1·02**	**1708·93******

Thorax length (TL) was included in all models as an index of body size. Standardized partial regression coefficients (β) and *F*-ratios are shown for each effect. Significant coefficients are highlighted in bold.
Error d.f. = 85, whole-model adjusted $R^2 > 0{\cdot}98$, $P < 0{\cdot}0001$.
$^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

of carbohydrate. Qualitatively similar results (not shown) were obtained for male total head length (head capsule + antenna). In contrast, we detected no significant effects of either protein, carbohydrate or their interaction on the relative dimensions of any of the legs or the wings (Table 4). Because the absolute sizes of all morphological traits exhibited qualitatively identical responses to macronutrient concentration (see above), these results indicate that, as dietary protein concentration increased, the growth rate of the larval tissues that give rise to the adult male head increased, relative to the growth of the rest of the body.

To visualize the effect of dietary protein on head elongation relative to body size, we plotted male residual total head length as a function of dietary protein. This reveals a complex pattern (Fig. 5). Although head elongation is reasonably well described by a quadratic function of protein, the data suggest a very sharp increase in head elongation from very low to moderate protein concentration, followed by a leveling-off at intermediate protein concentrations, and a further increase at very high protein concentration. After correcting for body size, the difference in total head length between males reared on the highest and lowest protein concentrations was about 2·25 standard deviations.

Discussion

Although it has long been recognized that secondary sexual traits tend to be highly condition-dependent (McAlpine 1979; Nur & Hasson 1984; Cotton, Fowler & Pomiankowski 2004), and are known to be sensitive to variation in nutrient availability (Hunt *et al.* 2004; Hall, Bussiere & Brooks 2008; Maklakov *et al.* 2008; South *et al.* 2011), we demonstrate for the first time (to our knowledge) that the development of adult male morphological structures that serve secondary sexual functions is especially sensitive to dietary protein. Increasing protein content in the larval diet of *Telostylinus angusticollis* resulted in enhanced expression of the most exaggerated and sexually dimorphic trait – male head shape. In contrast, neither protein nor carbohydrate content in the larval diet affected the relative dimensions of male legs or wings, suggesting that the effect of protein on body shape is limited to structures whose expression is most directly targeted by sexual selection. High protein content in the larval diet also enhanced adult male body size. However, increasing protein content in the larval diet was associated with a steep reduction in egg-to-adult survival probability. Our findings reveal novel effects of dietary protein on the development of male secondary

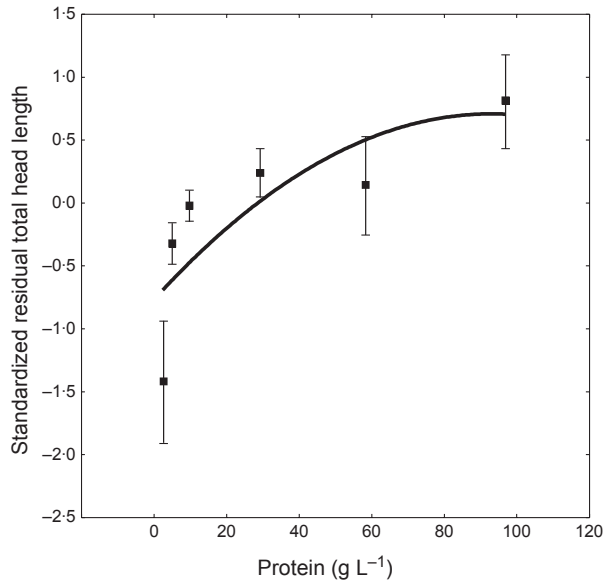

Fig. 5. Male residual total head (head capsule + antenna) length as a function of protein content (g L^{-1}) in the larval diet (see Materials and methods for details). Means for each level of protein are shown in standard deviation units, with bars representing the standard error of the mean of replicate means. A quadratic function is fitted to the data ($Y = -0{\cdot}77 + 0{\cdot}032X - 0{\cdot}00017X^2$).

sexual traits, and suggest that dietary protein mediates trade-offs across ontogenetic stages.

We detected some effects of carbohydrate concentration on larvae and adults, although these effects were generally weaker than the effects of protein. For viability, we detected a positive linear effect of carbohydrate, in contrast with the stronger negative linear effect of protein. We also detected a significant negative carbohydrate × protein interaction effect for this trait. Like protein, increasing carbohydrate concentration enhanced adult male body size, but the effect levelled off and eventually reversed at high concentrations. Interestingly, in contrast to protein, carbohydrate concentration in the larval diet had no linear or quadratic effects on male secondary sexual trait expression (indeed, the non-significant linear effects of carbohydrate on head capsule and antenna length are negative), although we detected a carbohydrate × protein interaction for head width. These complex and partly antagonistic effects are consistent with evidence from *Drosophila*, in which dietary protein and carbohydrate have opposing effects on at least one target of the insulin/IGF signalling pathway (Buch *et al.* 2008).

We have not yet examined the consequences of this range of larval diets for adult performance (although results for 'rich' and 'poor' larval diets differing in total nutrient concentration but not protein:carbohydrate ratio are reported in Bath, Tatarnic & Bonduriansky 2012; Adler *et al.* 2013, and unpublished manuscripts). However, our findings suggest that a protein-rich larval diet is likely to enhance adult male performance in *T. angusticollis* through its positive effects on growth and secondary sexual trait expression. Large body size allows males to defeat rivals in contests for control of advantageous territories and access to females (Bonduriansky & Head 2007). Large body size is also likely to confer survival advantages. For example, large *T. angusticollis* adults are more resistant to starvation (Adler *et al.* 2013). Yet, males reared on a high-protein diet were not only larger, but had longer and wider head capsules and longer antennae, both in absolute terms and after correcting for body size, than males provided with less dietary protein as larvae. Given the tight scaling of head and antenna dimensions with body size in this species (Bonduriansky 2006), this effect is surprisingly large: at a given body size, the total head length of males reared on the highest protein concentration exceeded that of males reared on the lowest protein concentration by over two standard deviations. The head is used as a weapon in male–male combat (Bonduriansky 2006), and head shape is strikingly sexually dimorphic (Bonduriansky 2007). Moreover, after correcting for larval diet (rich or poor) and body size, males bearing relatively elongated antennae are more likely to dominate rival males and have reduced latency to mating in no-choice pairings with females (C. Fricke, M.I. Adler, R.C. Brooks and R. Bonduriansky, unpublished data). Thus, head elongation appears to be under positive sexual selection in males. However, the development and expression of enlarged secondary sexual traits may reduce adult viability. Although the viability costs of these traits for adults are unknown in *T. angusticollis*, there is ample evidence for such costs in other species. For example, horned male beetles (*Onthophagus taurus*) have reduced ability to manoeuver through tunnels in the soil (Moczek & Emlen 2000) and horn expression trades off against the expression of adjacent morphological structures (Emlen 2001). Likewise, male stalk-eyed flies (*Cyrtodiopsis dalmanni*) and male rhinoceros beetles (*Trypoxylus dichotomus*) apparently require compensatory structures to maintain flight performance despite expressing large secondary sexual traits (Ribak & Swallow 2007; McCullough, Weingarden & Emlen 2013).

The disproportionate effect of dietary protein on male head shape, relative to body size, is likely to reflect heightened sensitivity of head and antenna imaginal tissues of *Telostylinus angusticollis* males to insulin/IGF signalling. Protein in the larval diet stimulates the secretion of insulin and IGFs that promote cell growth and proliferation (Britton & Edgar 1998; Brogiolo *et al.* 2001; Britton *et al.* 2002). Protein also provides the building blocks that are essential for growth (Teleman 2009; Harrison, Woods & Robers 2012). Body shape may be controlled developmentally by differential sensitivity of somatic tissues to insulin/IGFs (Shingleton *et al.* 2007; Shingleton, Mirth & Bates 2008), and it was recently shown that the larval tissues that develop into adult secondary sexual traits may be especially sensitive to insulin/IGF signalling: perturbing the transcription of the insulin receptor by RNA interference resulted in a disproportionate reduction in horn expression

in male *Trypoxylus dichotomus* beetles (Emlen *et al*. 2012). In *T. angusticollis*, the strong effect of protein on all three measured components of male head shape contrasts with the lack of any detectable effect of protein on the relative sizes of other morphological traits (legs and wings), suggesting that imaginal tissues destined to develop into structures that are most directly targeted by sexual selection are especially sensitive to the insulin/IGF signals induced by dietary protein, while other morphological traits are no more sensitive than whole-body growth. Consistent with this, male head dimensions also exhibited weaker loadings on the first principal component than other male traits (except wing length), indicating that the head dimensions are more variable in relation to body size than most other morphological traits. Results for female traits (to be reported in a separate paper) also contrasted sharply with those for males. In particular, we observed an effect of dietary protein but not carbohydrate on the relative length of female wings, suggesting that females may allocate protein resources to enhance locomotory capacity.

Interestingly, there was no evidence of heightened sensitivity to protein for the fore-tibia. Although the fore-legs are employed as weapons in male–male combat, and exhibit positive static allometry (Bonduriansky 2006), fore-tibia length exhibits less pronounced sexual dimorphism and weaker condition dependence than head shape (Bonduriansky 2007), and there is no evidence of sexual selection on fore-tibia length (C. Fricke, M.I. Adler, R.C. Brooks and R. Bonduriansky, unpublished data). Fore-tibia length is therefore likely to be a less direct target of sexual selection than head shape. Our results thus suggest that heightened sensitivity to protein in the larval diet is only a feature of morphological traits that are directly targeted by sexual selection and strongly condition-dependent.

We found that the positive effect of protein in the larval diet on adult body size and secondary sexual trait expression is counteracted by severe viability costs during the feeding/developmental stage. Protein's effect on egg-to-adult viability is so strong that it may counteract the benefits of large body size and enhanced secondary sexual trait expression in *T. angusticollis* adults and result in net balancing selection on these traits. High protein consumption by adults may increase mortality rate via increased oxidative damage, disruption of signalling pathways or circadian systems, toxicity of nitrogenous waste and impaired immune function (reviewed in Simpson & Raubenheimer 2009). Some of these effects may also occur in larvae. However, a protein-rich larval diet has also been found in other species to enhance larval or adult immunity, such that larvae on such a diet may enjoy an advantage in the presence of pathogens and parasites (Cotter *et al*. 2011).

The negative effect of a protein-rich larval diet on egg-to-adult viability is consistent with the tendency for *T. angusticollis* adults reared on a nutrient-rich larval diet to suffer more rapid somatic deterioration with age than individuals reared on a nutrient-poor diet (M.I. Adler and R. Bonduriansky, unpublished data). These findings suggest that the large adult body size and enhanced development of male secondary sexual traits induced by a protein-rich larval diet may result in a more fragile phenotype, susceptible both to death prior to reproductive maturity and to rapid 'mechanical ageing' in adulthood (Finch 1990). Nonetheless, the development of such a phenotype on a protein-rich larval diet may represent a facultative strategy that allows larvae to take advantage of abundant but transient resources: if large body size or large secondary sexual traits allow for a high reproductive rate in a highly competitive adult environment, then net fitness may be maximized by investing in rapid growth and large secondary sexual traits. Given the extremely high mortality rate and short life expectancy faced by insects in natural populations (Bonduriansky & Brassil 2002; Kawasaki *et al*. 2008; Zajitschek *et al*. 2009), the advantage of a high reproductive rate in the first few days of adult life may outweigh the disadvantages of an elevated risk of larval mortality and increased rate of adult ageing.

Although the range of phenotypic variation generated by our larval diet manipulation encompasses the phenotypic variation observed in natural populations of this species (A. Sentinella, A. J. Crean & R. Bonduriansky, unpublished data), we do not yet know the mean or range of macronutrient concentrations in the 'natural' larval diets encountered by wild *T. angusticollis*. We also do not know to what extent the larval and adult diets tend to covary among habitat patches in the wild although, given that both larvae and adults feed on rotting tree bark and sap, some degree of positive correlation may be expected. Laboratory experiments show that carbohydrate (sugar) in the adult diet is essential for reproduction in both sexes, whereas protein in the adult diet strongly enhances female reproduction but has more subtle and environment-dependent effects on male reproduction (Adler *et al*. 2013). While carbohydrate provides energy for male sexual activity, abundant protein in the adult diet may enhance production of sperm and accessory gland proteins (ACPs) (Fricke, Bretman & Chapman 2008). It is therefore possible that males emerging from a protein-rich larval patch and thus bearing enlarged secondary sexual traits (involved in pre-copulatory sexual selection) will also tend to encounter a protein-rich adult diet that promotes heightened sperm and ACP production (involved in post-copulatory sexual selection). The availability of dietary protein could thus regulate the intensity of male sexual competition.

We show important effects of protein on fitness-related traits during development, with associated trade-offs between larval and adult ontogenetic stages. These results support the view that no single diet can optimize all fitness components (Cotter *et al*. 2011): rather, the optimal protein concentration in the larval diet may depend on the environment that adults will experience. For example, when population density is high and adults face strong reproductive competition, a high-protein diet that enhances adult body size and male secondary sexual trait

expression may be optimal. Conversely, if population density is low, less protein in the larval diet and correspondingly greater survival rate of immature stages may be preferable, despite reduced mean body size. Population density is likely to vary both seasonally and spatially, such that female flies may be able to anticipate the conditions that their offspring are likely to encounter as adults. It is unclear whether female flies can assess such factors, as well as the macronutrient content of different oviposition substrates, and facultatively adjust their oviposition site choice so as to optimize offspring fitness.

Given that metabolic pathways are highly conserved across eukaryotic diversity (Fontana, Partridge & Longo 2010; Kenyon 2010), our results could apply to distantly related taxonomic groups such as vertebrates. Like insects, mammals respond to a nutrient-rich diet by upregulating signalling pathways that promote reproduction, and the abundance of dietary protein as well as the protein:carbohydrate ratio may play particularly important roles in this effect (Fontana, Partridge & Longo 2010; Piper *et al*. 2011). At the same time, in rodents, high protein consumption appears to lead to tissue damage and accelerated ageing (Sanz, Caro & Barja 2004). High protein intake during development also appears to impose viability-related costs in mammals (Tarry-Adkins *et al*. 2007; Martin-Gronert *et al*. 2008), but it remains unclear whether abundant dietary protein during the growth and development phase promotes the expression of adult secondary sexual morphology. Such effects may be reduced in vertebrates because secondary sexual traits are typically not fixed at reproductive maturity, allowing individuals to compensate for nutrient deficiency in development via supplementary feeding or differential resource allocation in adulthood (Birkhead, Fletcher & Pellatt 1999). Protein in the adult diet could also affect secondary sexual trait expression, although studies that have tested for this in vertebrates (the pheasant *Phasianus colchicus* and the white-tailed deer *Odocoileus virginianus*) did not detect an effect (Asleson, Hellgren & Varner 1997; Smith *et al*. 2007). It is therefore possible that dietary protein affects the expression of adult secondary sexual morphology only in taxa where the growth of such traits is completed prior to the adult stage, although further research is clearly needed.

In this study, we examined the effects of the two major macronutrients – protein and carbohydrates. Yet, both natural and artificial diets contain many other components, some of which are present in trace amounts. A recent study on the Australian tephritid fly *Bactrocera tryoni* showed that minerals in the adult diet have important effects on lifespan in addition to and in combination with amino acids (Fanson & Taylor 2011a). Water content in the diet can also have important effects on fitness and can interact with nutrient concentrations (Je *et al*. 2009; Fanson, Yap & Taylor 2012). A complete picture of the effects of protein and carbohydrate concentration in the larval diet therefore requires examination of the effects of other diet components, an understanding of the contents of the natural larval diet to which the flies have adapted over many generations, and the ecology of larval feeding.

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